Membrane-Bound Regulators of Complement Activation in Uveal Melanomas
CD46, CD55, and CD59 in Uveal Melanomas

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Purpose. To identify the presence of membrane-bound regulators of complement activation (m-RCA) on uveal melanomas and uveal melanoma cell lines and to examine their role in the inhibition of complement-mediated lysis in vitro.

Methods. Immunohistochemistry and flow cytometric analysis with monoclonal antibodies directed against m-RCA CD46, CD55, and CD59 were applied to tissue sections of 10 uveal melanomas, three primary uveal melanoma cell lines, and one uveal melanoma metastatic cell line. A microcytotoxicity test was used for measuring antibody-dependent complement-mediated lysis.

Results. The tissue sections and all four uveal melanoma cell lines expressed CD46, CD55, and CD59. Complement-mediated lysis in the presence of human complement was increased after partial removal of the m-RCA CD55 and CD59 with phosphatidylinositol-specific phospholipase C from the uveal melanoma cell line 92-1.

Conclusions. These results demonstrate that CD46, CD55, and CD59 are expressed in uveal melanomas and that CD55 or CD59, or both, plays a role in resistance to complement-mediated cytotoxicity. The finding that m-RCA are expressed in uveal melanomas may have implications for the effectiveness of the anti-tumor response and in the therapeutic application of monoclonal antibodies directed against tumor-associated antigens. Invest Ophthalmol Vis Sci. 1996;37:1884–1891.

Membrane-bound regulators of complement activation (m-RCA) play an important role in the protection of host cells from antibody-directed complement-mediated lysis. They also may play a role in downregulating autologous humoral anti-tumor responses and may limit the therapeutic application of monoclonal antibodies.

Recent studies have shown the existence of several membrane-bound complement-regulatory proteins that inhibit complement activation on the cell surface on which they are expressed. Three important m-RCA that regulate different stages of complement activation are membrane cofactor protein (MCP, CD46), decay-accelerating factor (DAF, CD55), and homologous restriction factor 20 (HRF20, CD59). CD46 is a cofactor for factor I-mediated cleavage of C3b and C4b. CD55 binds to C3b and C4b and accelerates the decay of the C3/C5 convertase enzymes. CD59 inhibits the formation of the membrane attack complex by binding to C8 and C9, which prevents pore formation.

Recently, several investigators have postulated that complement-regulatory proteins may play a role in tumor cell resistance to complement-mediated cytotoxicity. CD46 expression was increased two to eight times on human leukemia cell lines in comparison with the normal cell counterparts. In a study by Cheung et al, it was found that the expression of CD55 differed on various human melanoma and neuroblastoma tumor cell lines. A relationship was shown between resistance to complement-mediated killing and CD55 expression. Blocking of CD55 with monoclonal antibodies increased the sensitivity of CD55-positive cell lines to lysis. The presence of m-RCA on...
tumor cells might play a role in downregulating the autologous anti-tumor response.

We were interested in the role of m-RCA with regard to ocular melanomas. Although no information is available on m-RCA expression in ocular melanomas, information is available about the expression of m-RCA in the different tissues of the human eye. Expression of various m-RCA have been observed in the cornea, ciliary body, iris, choroid, and retina, with more intense staining of CD59 than of CD46 and CD55, providing evidence that in the eye a complement regulatory system exists.8,9

In our study, we examined the role of m-RCA in uveal melanomas: We first determined the presence of the m-RCA by immunohistochemistry in snap-frozen tissue sections and by flow cytometric analysis of uveal melanoma cell lines with monoclonal antibodies directed against the m-RCA CD46, CD55, and CD59. The tissue sections of 10 uveal melanomas and the cell lines from three primary uveal melanomas and one metastasis were found to express these m-RCA. In addition, we examined the functional role of CD55 and CD59 in cultured uveal melanoma cells after the removal of these phosphatidylinositol-linked m-RCA with phosphatidylinositol-specific phospholipase C and determined the subsequent changes in sensitivity to complement-mediated lysis.

MATERIALS AND METHODS

Antibodies and Complement Sources

The following monoclonal antibodies were used: GB24 (IgG1) against CD46 (Theramec, Monaco, France)10,11; BRIC 110 (IgG1) against CD55 (BGRL Office, Bristol, UK)12,13; BRIC 229 (IgG2a against CD59 (BGRL Office)14; W6/32 (IgG2a) against anti-HLA class I (ATCC, Rockville, MD); Leu-4 (IgG1) against CD3 (Becton Dickinson, San Jose, CA)15; peroxidase-conjugated rabbit anti-mouse immunoglobulin antibodies and peroxidase-conjugated swine anti-rabbit immunoglobulin antibodies (DAKO, Glostrup, Denmark) for immunohistochemical staining; and fluorescein-conjugated rabbit anti-mouse immunoglobulin antibody (DAKO) for flow cytometry.

As complement sources we used pooled rabbit serum (Stichting Bioscience Foundation, Leiden, Netherlands) and normal human sera collected from healthy blood donors. Sera were aliquoted and frozen at —80°C and stored until use.

Histopathologic Examination

Tumor specimens were obtained from uveal melanomas (n = 10) immediately after enucleation. The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained from each patient before surgery after the nature of the study was explained. The diagnosis of each lesion was established using conventional histologic sections prepared from celloidin-embedded tissue stained with hematoxylin and eosin. The following parameters were determined in all cases: intraocular localization (choroid, ciliary body, or both), cell type (spindle, epithelioid, or mixed-cell type), largest tumor diameter and prominence, mitotic frequency (counted in 15 high-power fields), and scleral invasion (absent, slight, moderate, deep, or episcleral). The clinico-histopathologic data are shown in Table 1.

Immunohistochemistry

Part of the tumor was removed, snap frozen in liquid isopentane, and stored at —80°C until further use. A three-step immunoperoxidase technique was used as described previously.16 GB24 (3 µg/mL), BRIC 110 (3 µg/mL), and BRIC 229 (6 µg/mL) were used as primary antibodies. Peroxidase-conjugated rabbit anti-mouse immunoglobulin antibodies and peroxidase-conjugated swine anti-rabbit immunoglobulin antibodies were used as secondary and tertiary antibodies. The peroxidase reaction was developed using 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MO) in 0.1 M sodium acetate buffer (pH 5) containing 0.05% H2O2. Negative control stains were performed by omitting the primary antibodies. Slides were examined independently by two observers, who scored the staining intensity semiquantitatively and graded them from negative (−) to strongly positive (++++).

Cell Lines

Three cell lines obtained from human primary uveal melanomas (92-1, MEL-202, and OCM-1), and one cell line obtained from a human metastatic uveal melanoma (OMM-1) were used. Cell line 92-117 and OMM-118 were established in our own laboratories. Cell line MEL-202 was kindly provided by Dr. B. Ksander (Schepens Eye Research Institute, Harvard Medical School, Boston, MA). Cell line OCM-1 was a generous gift of Dr. J. Kan–Mitchell (University of California, San Diego). Cell lines 92-1 and MEL-202 were grown as monolayers in RPMI 1640 (Gibco, Paisley, Scotland) with 3 mM L-glutamine (Gibco), 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT), 100 IU/ml penicillin (Gibco), and 100 µg/mL streptomycin (Gibco); cell lines OMM-1 and OCM-1 were grown in Dulbecco's modified Eagle's medium (Gibco) with Hapes buffer (25 mM), sodiumpyruvate (1 mM), 1 g/l glucose, 10% FCS (Hyclone), and 100 IU/ml penicillin and 100 µg/mL streptomycin (Gibco). Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

Flow Cytometry

Single-cell suspensions were made by detachment of the cells using 0.01% trypsin in phosphate-buffered saline.
TABLE 1. Clinical and Histopathologic Data of Uveal Melanomas

<table>
<thead>
<tr>
<th>Tumor Number</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Location</th>
<th>Cell Type</th>
<th>Diameter (mm)*</th>
<th>Prominence (mm)*</th>
<th>Mitotic Frequency†</th>
<th>Scleral Invasion</th>
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<tr>
<td>1</td>
<td>59</td>
<td>F</td>
<td>Choroid</td>
<td>Spindle</td>
<td>10</td>
<td>9</td>
<td>21</td>
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<td>7</td>
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<td>—</td>
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<tr>
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<td>77</td>
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<td>Giliary body-choroid</td>
<td>Spindle</td>
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<td>6</td>
<td>13</td>
<td>Absent</td>
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<tr>
<td>7</td>
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<td>F</td>
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<td>Spindle</td>
<td>12</td>
<td>5</td>
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<td>Deep</td>
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<td>8</td>
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<td>Mixed</td>
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<td>10</td>
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<td>6</td>
<td>12</td>
<td>Slight</td>
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<tr>
<td>10</td>
<td>77</td>
<td>F</td>
<td>Giliary body-choroid</td>
<td>Spindle</td>
<td>11</td>
<td>10</td>
<td>3</td>
<td>Slight</td>
</tr>
</tbody>
</table>

* Largest diameter and prominence of the tumor as assessed by histopathologic examination.
† Number of mitoses per 15 fields of microscopic examination (magnification, ×320).

(PBS). After washing in PBS–bovine serum albumin (BSA) 1%, 50 μl cell suspensions containing 0.1 × 10⁶ cells were incubated with 50 μl dilutions of either GB24 (2 μg/ml), BRIC 110 (3 μg/ml), BRIC 229 (6 μg/ml), or W6/32 (10 μg/ml). Thereafter, the cells were washed in PBS–BSA 1%, which was followed by incubation with fluorescein-conjugated rabbit anti-mouse immunoglobulin antibody (DAKO) for 1 hour. All incubations were performed at 4°C. Flow cytometric measurements were performed with a FACScan II (Becton Dickinson). Statistical analyses were performed using the LYSYS II software (Becton Dickinson). Background fluorescence was determined by using an irrelevant primary monoclonal antibody (Leu-4).

Complement-Mediated Cytotoxicity Assay

A double-color fluorescent microcytotoxicity test, normally used for HLA-typing, was used for measuring antibody-dependent, complement-mediated lysis on cell line 92-1 and peripheral blood lymphocytes (PBL). These PBL were obtained from one healthy blood donor and were isolated by Ficoll separation and cryopreserved in liquid nitrogen until use. Approximately 1 × 10⁶ cells, either 92-1 melanoma cells or PBL, were incubated for 10 minutes with 1.5 ml (25 μg/ml) carboxyfluoresceine-di-acetate label at room temperature in the dark. Labeled cells were counted and distributed (2500 cells/well, 1 μl) in 20 μl micro-wells of Terasaki microtest trays containing 8 μl oil per well. After incubation with various concentrations (0.25 to 4 μg/ml; 1 μl) W6/32 (30 minutes), each well was incubated with 5 μl pooled rabbit serum or normal human serum in various dilutions (12.5%, 25%, 50%, and 100% for 45 minutes). Finally, to each well, 10 μl solution of propidium iodide (Sigma) and quenching ink (Leica, Rijswijk, Netherlands) was added.

TABLE 2. Expression of CD46, CD55, and CD59 in Snap-Frozen Tissue Sections of Uveal Melanomas, as Determined by Immunohistochemistry

<table>
<thead>
<tr>
<th>CD46</th>
<th>CD55</th>
<th>CD59</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>E</td>
<td>T</td>
</tr>
<tr>
<td>1</td>
<td>+ /-</td>
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</tr>
<tr>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>+ /-</td>
<td>+ /-</td>
</tr>
</tbody>
</table>

Comparative staining intensities were graded as follows: - = negative; + /- = rarely positive; + = weakly positive; ++ = moderately positive; +++ = strongly positive. T = tumor cells; E = endothelium of vessels in tumor.

* Tumor identification number.
Membrane-Bound Regulators in Uveal Melanomas

HOMM-1
OCM-1
92-1
MEL 202
CD46
CD55
m-RCA
CD59

FIGURE 1. Flow cytometric analysis of three primary uveal melanoma cell lines (OCM-1, 92-1, and MEL-202) and one cell line grown from a uveal melanoma metastasis, stained with monoclonal antibodies against CD46, CD55, and CD59. Results are expressed as the mean fluorescence intensity.

added. The fluorescence intensity was measured after 10 minutes in the dark with an automated fluorescence microscope (Patimed; Leica). The percentage of lysis was determined relative to the lysis achieved with 50% diluted pooled rabbit serum and 1 µg/ml W6/32 at each test for both cell line 92-1 and PBL. Background lysis was obtained by incubation of the cells with RPMI 1640 containing 10% FCS only.

Interferon-γ Treatment
Monolayers of cell line 92-1, approximating $3 \times 10^6$ cells, were grown to confluency in a petri dish, and PBL, approximating $10 \times 10^6$ cells, were resuspended in 10 ml RPMI 1640 supplemented with 10% FCS and 20 U/ml IL-2 (Cetus, Emeryville, CA). Both cell line 92-1 and PBL were incubated with 12.5, 50, or 200 U/ml recombinant human interferon-γ (IFNγ) for 48 hours. IFNγ was kindly provided by Dr S. Osanto (Leiden University Hospital, Netherlands). Medium alone served as control.

Phosphatidylinositol-Specific Phospholipase C Treatment
Cell suspensions of cell line 92-1 or PBL were washed twice with RPMI 1640 supplemented with BSA 1%. In this medium, the cells ($2 \times 10^6$ cells/ml) were resuspended and incubated for 1 hour at 37°C with 100 mU/ml Bacillus thuringiensis, phosphatidylinositol-specific phospholipase C (PIPLC; ICN Biomedicals, Zoetermeer, Netherlands). Cells were washed twice with this medium and used for further experiments.

RESULTS
Immunohistochemical Detection of CD46, CD55, and CD59 in Uveal Melanoma Tissue Sections
We determined the presence of CD46, CD55, and CD59 in tissue sections of 10 uveal melanomas obtained after enucleation of the eye. We applied monoclonal antibodies specific for three different m-RCA—i.e., for CD46, CD55, and CD59. All tumors displayed a similar expression: Tumor cells stained strongly positive for CD59 but only moderately positive for CD55 and weakly positive for CD46, with some tumors not expressing CD46 at all (Table 2). Expression of m-RCA on endothelium of blood vessels in the tumor usually showed the same level of expression as the surrounding tumor tissue.

Flow Cytometric Analysis of CD46, CD55, and CD59 on Uveal Melanoma Cell Lines
To have a more quantified measurement of m-RCA in uveal melanomas, we also determined the level of expression on uveal melanoma cell lines using flow cytometry. The level of expression of CD46, CD55, and CD59 was determined on four uveal melanoma cell lines (Fig. 1). We compared the mean fluorescence intensity after subtraction of background fluorescence intensity using a control antibody. Similar to the results on uveal melanoma sections, CD59 staining was the most intense; CD46 and CD55 were expressed at lower levels. CD55 expression especially varied between cell lines.

Influence of m-RCA on Antibody-Dependent, Complement-Mediated Lysis
Because we wanted to determine the functional role of m-RCA on uveal melanomas, we measured the anti-

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body-dependent, complement-mediated lysis of one uveal melanoma cell line (cell line 92-1). Human m-RCA, especially CD59, have a species-restricted effect, and their blocking effect can be overcome by using complement from a different species in the assay system. In this study, we chose the W6/32 antibody (IgG1, directed against HLA class I, as a model for a tumor-associated antigen) to induce complement activation and to investigate the role of CD55 and CD59 on the level of lysis. As a positive control in this system, PBL were used for comparison. In the presence of rabbit serum (50%) and 0.25 ng/ml W6/32, only 72% lysis of 92-1 cells was measured. At 0.5 μg/ml or 1 μg/ml W6/32, more than 97% lysis of the 92-1 tumor cell line was achieved. PBL were lysed 100% at each concentration W6/32 tested.

Because we wanted to know whether m-RCA played a role in the inhibition of complement-mediated lysis of human uveal melanoma cells, we removed the phosphatidyl-inositol-linked CD55 and CD59 of the tumor cells with PIPLC. As shown in Figure 2, almost all CD55 and CD59 molecules were removed from the cell surface. We subsequently determined the effect of PIPLC treatment on complement-mediated lysis.

No measurable lysis was obtained when 92-1 cells were incubated with normal human serum as the source of complement (with or without previous PIPLC treatment) and 0.25 to 4 μg/ml W6/32 (Fig. 3), whereas PBL were lysed up to 70% under similar conditions (Fig. 4). The absence of lysis of 92-1 cells, despite the removal of the majority of CD55 and CD59 (Fig. 2), suggested that the expression of HLA class I on 92-1 cells was too low to bind enough W6/32 to activate the complement system.

Previous experiments had shown that the addition of IFNγ induced a higher expression of HLA class I on 92-1 cells. Indeed, flow cytometric analysis showed that the expression of HLA class I on uveal melanoma cells was lower than on PBL and could be increased for 92-1 cells and PBL after incubation with IFNγ (Table 3). Treatment with PIPLC did not diminish HLA class I expression on 92-1 cells or PBL.

Finally, we repeated the cytotoxicity experiments after incubation of the tumor cells with IFNγ. Now lysis occurred in 92-1 cells (Fig. 3) as well as in PBL (Fig. 4) with 1 to 4 μg/ml W6/32. Treatment with PIPLC, which reduced the expression of CD55 and CD59 on 92-1 cells and PBL after treatment with IFNγ (Table 3), resulted in increased lysis for 92-1 and PBL (Figs. 3, 4), indicating that either CD55 or CD59, or both, is important in the protection of uveal melanoma cells against complement-mediated lysis.

**DISCUSSION**

The aim of our study was to investigate whether uveal melanoma cells express CD46, CD55, and CD59 and whether such molecules help to protect uveal melanoma cells against complement-mediated lysis. Immuno-
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FIGURE 4. Lysis of peripheral blood lymphocytes (PBL) after treatment with IFNγ and phosphatidylinositol-specific phospholipase C (PIPLC). Lysis was measured in an antibody-dependent, complement-mediated cytotoxicity assay using W6/32 as an antibody and normal human serum as a source of complement. Without PIPLC treatment lysis was observed at W6/32 concentrations starting at 0.5 μg/ml. Treatment with PIPLC, which reduces the expression of CD55 and CD59, resulted in increased lysis of PBL at W6/32 concentrations starting at 0.25 μg/ml. Treatment with IFNγ resulted in increased lysis of PBL, both treated with or without PIPLC. Results represent one of three similar independent experiments.

TABLE 3. Expression of HLA Class I, CD46, CD55, and CD59 on 92-1 Uveal Melanoma Cells and PBL

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Fluorescence Intensity</th>
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<tbody>
<tr>
<td>IFNγ</td>
<td>PIPLC</td>
</tr>
<tr>
<td>92-1</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
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<tr>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PBL</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
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<tr>
<td>−</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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</tbody>
</table>

The fluorescence intensity was determined by flow cytometry, after subtracting background lysis, using an irrelevant monoclonal antibody. The effects of treatment with (+) or without (−) IFNγ and/or PIPLC on the mean fluorescence intensity is shown.

PBL = peripheral blood lymphocytes; PIPLC = phosphatidylinositol-specific phospholipase C.
have shown expression of CD59 in human metastatic melanoma lesions and demonstrated that CD59 was an important factor in protecting melanoma against homologous complement-mediated lysis. Our cytotoxicity tests support a role for CD55 or CD59, or both, in increasing the complement-mediated lysis on the 92-1 cells because removal of CD55 and CD59 by PIPLC leads to enhanced complement-mediated lysis. We found that CD59 was highly (and homogeneously) expressed in the uveal melanoma tumor sections, as well as on the uveal melanoma cell lines. The expression pattern is similar to the high expression of CD59 observed on most skin melanoma cell lines. This suggests that CD59 is an important m-RCA in uveal melanomas.

An important difference exists in how Brasoveanu et al. and we set up our cytotoxicity assays. They used an anti-GD3 ganglioside monoclonal antibody to activate the complement system, whereas we used an anti-HLA class I monoclonal antibody (W6/32). Using W6/32 and human serum, we were at first unable to induce complement-mediated lysis of our uveal melanoma cell line. Skin and uveal melanoma cells have been shown to have a low expression of HLA class I molecules. Indeed, the expression of HLA class I molecules on the uveal melanoma cell line 92-1 was approximately 35% of the level expressed by PBL. When we increased the expression of HLA class I molecules on 92-1 by IFNγ, lysis was established and we were able to show an effect of PIPLC on the complement-mediated lysis of the tumor cells. The absence of lysis of 92-1 cells after the removal of the majority of CD55 and CD59 with PIPLC and the induction of lysis of 92-1 cells after treatment with IFNγ and PIPLC suggested that lysis depended on a critical number of antigenic determinants. It should be noted that higher amounts of IFNγ (e.g., 200 U/ml, not shown) seemed to decrease the expression of CD59 without affecting CD46 or CD55. At present, we are investigating the effect of cytokines such as IFNα, interleukin 1β, and tumor necrosis factor α on the modulation of the expression of m-RCA and the effect on complement-mediated lysis (manuscript in preparation). In a study on the influence of various cytokines on the expression and function of CD59 on a human colonic adenocarcinoma cell line, CD59 expression was increased by low doses of IFNγ in a dose-dependent fashion and was partially responsible for an increased resistance to lysis compared to untreated cells. However, in the study of the skin melanoma cell lines, treatment of the tumor cells with IFNγ neither modulated the expression of CD59 on melanoma cells nor influenced the amounts of CD59-specific mRNA.

The finding that m-RCA are expressed in uveal melanomas may have implications in anti-tumor responses and in the therapeutic application of monoclonal antibodies. It has been shown that tumor-associated antibodies occur in patients with uveal melanomas and did not prevent tumor development and tumor growth. This phenomenon already has been observed in a wide variety of patients with cancer. Future studies may determine whether m-RCA inhibit autologous anti-tumor responses in uveal melanomas.

Finally, it remains to be determined how the complement system is regulated within the environment of the eye. Expression of various m-RCA has been observed in the cornea, ciliary body, iris, choroid, and retina, with more intense staining of CD59 than of CD46 and CD55. In our study, CD59 was most dominantly expressed on sections of uveal melanomas, on three primary uveal melanoma cell lines, and on one metastatic cell line. Nevertheless, functional analysis of the m-RCA by cytotoxicity assays showed an important role for CD55 or CD59, or both, in the protection of uveal melanomas against antibody-dependent, complement-mediated lysis. Future studies on the influence of various cytokines on the expression and function of m-RCA in uveal melanomas might help in designing effective therapeutic anti-tumor strategies.

Key Words
CD46, CD55, CD59, complement, complement regulatory proteins, cytotoxicity assay, immunohistochemistry, uveal melanomas

Acknowledgments
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